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Type III Secretion System and Virulence Markers Highlight Similarities and Differences between Human- and Plant-Associated Pseudomonads Related to *Pseudomonas fluorescens* and *P. putida*

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***Pseudomonas fluorescens* is commonly considered a saprophytic rhizobacterium devoid of pathogenic potential. Nevertheless, the recurrent isolation of strains from clinical human cases could indicate the emergence of novel strains originating from the rhizosphere reservoir, which could be particularly resistant to the immune system and clinical treatment. The importance of type three secretion systems (T3SSs) in the related *Pseudomonas aeruginosa* nosocomial species and the occurrence of this secretion system in plant-associated *P. fluorescens* raise the question of whether clinical isolates may also harbor T3SSs. In this study, isolates associated with clinical infections and identified in hospitals as belonging to *P. fluorescens* were compared with fluorescent pseudomonads harboring T3SSs isolated from plants. Bacterial isolates were tested for (i) their genetic relationships based on their 16S rRNA phylogeny, (ii) the presence of T3SS genes by PCR, and (iii) their infectious potential on animals and plants under environmental or physiological temperature conditions. Two groups of bacteria were delineated among the clinical isolates. The first group encompassed thermotolerant (41°C) isolates from patients suffering from blood infections; these isolates were finally found to not belong to *P. fluorescens* but were closely related and harbored highly conserved T3SS genes belonging to the Ysc-T3SS family, like the T3SSs from *P. aeruginosa*. The second group encompassed isolates from patients suffering from cystic fibrosis; these isolates belonged to *P. fluorescens* and harbored T3SS genes belonging to the Hrp1-T3SS family found commonly in plant-associated *P. fluorescens*.**

The group of fluorescent pseudomonads is composed of ubiquitous bacteria commonly encountered in aquatic, aerial, and soil environments (1, 2). Under iron limitation, members of this group share the ability to produce soluble greenish fluorescent siderophores that appear fluorescent under UV illumination (3). These bacteria display a broad potential for adaptation to fluctuating environmental conditions, such as temperature variations, thanks to their highly versatile metabolism and the plasticity of their large genome (4–6). Some species in this group, such as *Pseudomonas fluorescens*, are commonly associated with plants as saprotrophs, especially in the rhizosphere since their densities and activities are greater than in the surrounding soil (6–9). Some of these populations may have beneficial effects on plant growth and health (10). In contrast, other fluorescent *Pseudomonas* species are pathogens of humans, animals, and plants, i.e., eukaryotic hosts (11, 12).

The type three secretion systems (T3SSs) have been shown to play a determining role in the pathogenicity produced by fluorescent pseudomonads such as *P. aeruginosa* and *P. syringae*, pathogens of humans and plants, respectively (11). In these two pathogenic species, T3SS is involved in cell-to-cell contact with the eukaryotic host and in bacterial virulence. Genes encoding the basic structure of the T3SS are conserved among Gram-negative bacteria (13, 14). Based on sequence analysis, eight families of T3SSs have been described (15, 16). In the human pathogen *P. aeruginosa*, T3SSs belong to the Ysc-T3SS family; they form a short needle embedded within the two Gram-negative bacteria membranes, which serves as a secretion channel for the injection of exoenzymes, playing key roles in eukaryotic cell intoxication, into the target cell cytosol (17–19). In the plant pathogen *P. syringae*, the T3SS involved in plant pathogenicity belongs to the Hrp1-T3SS family; it produces a long and flexible pilus, similar to a flagellum, which is capable of transpiercing the thick wall of plant cells and allows the injection of multiple effector proteins into the cells that suppress the plant's innate immune defenses, manipulate hormone signaling, and elicit cell death (20, 21).

Although the *P. fluorescens* species is considered nonphytopathogenic, T3SS genes related to the Hrp1-T3SS family (15, 16) have been reported in rhizosphere populations (22–24). More generally, the presence of this secretion system involved in cell-to-cell interactions with eukaryotic organisms appears to be quite a

Received 19 December 2014 **Accepted** 22 January 2015

Accepted manuscript posted online 30 January 2015

Citation Mazurier S, Merieau A, Bergeau D, Decoin V, Sperandio D, Crépin A, Barbey C, Jeannot K, Vicré-Gibouin M, Plésiat P, Lemanceau P, Latour X. 2015. Type III secretion system and virulence markers highlight similarities and differences between human- and plant-associated pseudomonads related to *Pseudomonas fluorescens* and *P. putida*. Appl Environ Microbiol 81:2579–2590. doi:10.1128/AEM.04160-14.

Editor: M. Kivisaar

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doi:10.1128/AEM.04160-14

common feature in nonpathogenic strains of plant-associated *P. fluorescens* (16, 23, 25).

Also, although considered nonpathogenic to humans, *P. fluorescens* isolates have been isolated in hospitals from clinical water or humid environments (e.g., sinks, drains, toilet linen, and objects) and from patients, in which they may be part of the normal epidermal and oropharyngeal flora (26–28). Most of these isolates are psychrotrophic and able to resist many antibiotics and antiseptics, and they can therefore alter patient food, biological reagents, and blood injecting solutes or derivatives usually stored under refrigeration (29–37). Finally, there is increasing evidence that some *P. fluorescens* strains may be pathogenic for humans or animals. This bacterium has indeed been involved in cases of nosocomial infections, e.g., infections of the urinary tract in immunocompromised patients and of the respiratory tract in a patient with lung cancer (38, 39). *P. fluorescens* strains have also been reported to be the causative agents of septicemia, peritonitis, and lethal liver infections and are considered putative opportunistic pathogens (40–45).

The implication of T3SSs in the virulence of pathogenic fluorescent pseudomonads and the presence of T3SSs in plant-associated *P. fluorescens*, taken together, raise questions about the possible presence of T3SSs in clinical *P. fluorescens* and a possible link between these T3SSs and the infectious potential of the corresponding isolates.

In this study, pseudomonads associated with clinical infections and initially identified as *P. fluorescens* in hospitals were compared with plant-associated isolates of *P. fluorescens* harboring T3SSs and with reference strains. These isolates and strains, suspected to be opportunistic pathogens, were compared for a series of genetic and phenotypic traits: (i) 16S rRNA phylogeny; (ii) presence of T3SS genes belonging to Hrp1 and Ysc, two distinct T3SS families commonly observed in plant-associated strains of pseudomonads and in the human pathogen *P. aeruginosa*, respectively; (iii) phylogeny of these T3SS genes; and (iv) infectious potential on animals and plants under environmental and physiological temperatures.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Characteristics of the *Pseudomonas* isolates and references used in this work are presented in Table 1. A first set of 12 strains were collected in hospitals in two French regions distant from each other (Franche-Comté and Normandy) from 12 patients, 6 suffering from pulmonary tract infections and 6 from blood infections (bacteremia). Each strain tested was the only one isolated for each blood sample and was predominant for each expectoration sample. In the hospitals, they had been originally identified as *P. fluorescens* on the basis of the API 20 NE test (bioMérieux S.A., Craponne, France). None of these isolates produced the pyocyanin pigment in King A medium or the quorum-sensing *Pseudomonas* quinolone signal (data not shown), traits shared only by *P. aeruginosa* strains (46, 47). A second set of 12 strains associated with plants consisted of (i) 3 reference strains harboring T3SSs, i.e., C7R12, SBW25, and F113, well described for their beneficial effects on plant growth and health and for the presence of T3SSs (22, 23, 48), and (ii) 9 additional strains isolated from the rhizosphere or phyllosphere of four plant species and chosen to cover the diversity of *hrcRST* (*rscRST*) genes described so far in *P. fluorescens* (23, 49). A third set was composed of three reference strains and included (i) the type strain of *P. fluorescens* species (ATCC 13525^T type strain), which does not harbor any T3SS gene, and (ii) two pathogenic strains of *Pseudomonas* extensively studied for their T3SSs, one strain pathogenic to humans, *P. aeruginosa* PAO1, and one strain pathogenic to plants, *P. syringae* pv. tomato DC3000 (50–52). Finally, the strains *Klebsiella aerogenes* (53) and *P. aeruginosa* UCBPP-PA14 (here referred to as *P. aeruginosa* PA14) were used as controls for the detection of virulence against the *Dictyostelium discoideum* model (54).

Fluorescent pseudomonad cultures were grown in liquid or solid King's B (KB) medium (46) at 4, 25, 37, and 41°C, a range of temperatures covering environmental conditions and those of warm-blooded animals. *K. aerogenes* was grown in Luria-Bertani (LB) medium at 37°C. Results are mean values from at least three independent experiments.

PCR amplifications, DNA sequencing, and phylogenetic analysis. PCR amplifications were carried out on total DNA extracted using standard methods and primers synthesized by Eurogentec (Angers, France). *hrcRST* (*rscRST*) and *pscRST*, genes encoding structural components of T3SSs belonging to the Hrp1 and to the Ysc family, respectively, were amplified. *hrcRST* (*rscRST*) DNA fragments were amplified using HRCR8092 5′-CCITT(C/T)ATCGT(C/T)AT(C/T)GA(C/T)(C/T)T-3′ and HRCT8986 5′-CTGTCCCAGATIAICTGIGT-3′ (where I indicates d-inosine), as previously described by Mazurier et al. (23). *pscRST* DNA fragments were amplified with HRCRPa, 5′-CCNTT(C/T)ATCGAT (C/T)GA(C/T)C-3′, and HRCTPa, 5′-AC(A/C)GGCCAACTTGS(A/G) TAGC-3′. These primers were designed on the basis of alignments of *P. aeruginosa* PAO1, UCBPP-PA14, and LESB58 sequences corresponding to *hrcRST* sequences targeted by HRCR8092 and HRCT8986, respectively. *hrcRST* (*rscRST*) and *pscRST* PCR amplifications were performed in a total volume of 25 μl containing 20 ng of purified DNA, each primer at a concentration of 0.5 μM, with 50 μM dATP, 50 μM dTTP, 50 μM dGTP, 50 μM dCTP, and 2 U of *Taq* DNA polymerase (Q-BIOgene, Illkirch, France) in the PCR buffer (10 mM Tris-HCl [pH 9.0 at 25°C], 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% [vol/vol] Triton X-100, 0.2 mg/ml bovine serum albumin [BSA]; Q-BIOgene, Illkirch, France). Amplifications were conducted in a PTC-200 thermal cycler (MJ Research, Waltham, MA) by using an initial denaturation step consisting of 3 min at 95°C, followed by 35 cycles of 1 min at 94°C, 1 min at 67°C, and 2 min at 72°C before a final elongation for 5 min at 72°C. The size of the PCR products was checked by electrophoresis in 1% agarose gels. PCR bands of the expected size (ca. 900 bp) were excised and extracted from the gel by using the Montage gel extraction kit (Millipore, Molsheim, France) as recommended by the manufacturer. PCR fragments were cloned (pGEMT Easy Vector System II; Promega, Charbonnières, France) and sequenced (Eurofins MWG Operon, Ebersberg, GeCrmany). PCR amplification of the 16S rRNA gene was performed commercially by Macrogen Inc. (Seoul, South Korea) using the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′), 518F (5′-CCAGCAGCCGCGGTAAT-3′), 800R (5′-TACCAGGGTATC TAATCC-3′), and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) within the framework of the "16S rRNA gene full sequencing" service. BLAST analyses were performed, DNA sequences were aligned, and phylogenetic trees were reconstructed by the neighbor-joining method (55) as implemented in ClustalW2 (56). DNA distances were calculated with Kimura's two-parameter correction (57).

cHA assay. A cell-associated hemolytic activity (cHA) assay was performed as described by Sperandio et al. (58). Briefly, sheep red blood cells (RBCs; Eurobio, Courtaboeuf, France) were washed three times in phosphate-buffered saline (PBS) (pH 7.2), consisting of NaCl (0.8%, wt/vol), KCl (0.02%, wt/vol), Na_2HPO_4 (0.17%, wt/vol), KH_2PO_4 (0.8%, wt/vol), and resuspended in RPMI 1640 medium without pH indicator (Sigma-Aldrich, Saint-Quentin Fallavier, France) at a density of 5×10^8 RBCs/ml at 4°C. Bacteria were grown in KB medium to an optical density at 580 nm (OD_{580}) of 0.7 to 1 at 25°C or 37°C, centrifuged, and resuspended in RPMI 1640 at 5×10^8 bacteria/ml. Assays were started by mixing the RBC suspension (100 μl) and the bacterial cell suspension (100 μl), which were then centrifuged at 400 × *g* for 10 min and incubated at 37°C for 1 h. The release of hemoglobin was measured at 540 nm, after centrifugation, in 100 μl of supernatant. The percentage of total cell-associated hemolytic activity was calculated as follows: $\% = [(X - B)/(T - B)] \times 100$, where *B* (baseline), a negative control, corresponds to the number of RBCs incubated with 100 μl of RPMI 1640, and *T*, a positive control, corresponds to

TABLE 1 Characteristics of the fluorescent pseudomonads studied and results of T3SS marker genes' detection by PCR

Strain (synonym)	Geographic origin (yr of isolation)	Host or source of isolation	PCR detection of T3SS genes[a]: *pscRST*	PCR detection of T3SS genes[a]: *hrcRST (rscRST)*	Reference or source
Human pathogen reference strain *P. aeruginosa* PAO1	Australia (1955)	Burned patient	+	−	90
Pseudomonas fluorescens hospital strains					
B-Hemo1 (Hé1)	Franche-Comté hospital (2006)	Blood culture (bacteremia), patient 1	+	−	This study
B-Hemo2 (00099)	Franche-Comté hospital (2007)	Blood culture (bacteremia), patient 2	+	−	This study
B-Hemo3 (96244)	Franche-Comté hospital (2005)	Blood culture (bacteremia), patient 3	+	−	This study
B-Hemo4 (GI2004)	Franche-Comté hospital (2004)	Blood culture (bacteremia), patient 4	+	−	This study
N-Hemo5 (MFY161)	Normandy hospital (2003)	Blood culture (bacteremia), patient 5	+	−	85
N-Hemo6 (MFY163)	Normandy hospital (2003)	Blood culture (bacteremia), patient 6	+	−	This study
N-Absc7 (MFY70)	Normandy hospital (2003)	Abscess (bacteremia), patient 7	−	+	85
B-Exp8 (4286)	Franche-Comté hospital (2006)	Expectoration (cystic fibrosis), patient 8	−	+	This study
B-Exp9 (2967)	Franche-Comté hospital (2003)	Expectoration (cystic fibrosis), patient 9	−	+	This study
N-Exp10 (MFY162)	Normandy hospital (2003)	Expectoration (cystic fibrosis), patient 10	−	+	85
N-Exp11 (MFN1032)	Normandy hospital (2003)	Expectoration (cystic fibrosis), patient 11	−	+	91
N-Suc12	Normandy hospital (2003)	Pulmonary suction, patient 12	−	+	This study
Pseudomonas fluorescens type strain ATCC 13525^T (CFBP[b] 2102)	United Kingdom (1951)	Water tank	−	−	CFBP, type strain
Pseudomonas fluorescens plant strains					
C7R12	France, Châteaurenard, soil (1989)	*Linum usitatissimum*	−	+	92
F113 (CFBP 5935)	Ireland (1992)	*Beta vulgaris*	+	+	93
SBW25	United Kingdom (1994)	*Beta vulgaris*	−	+	94
M-Maiz1 (CFBP 5897[c])	France, Maine valley (1992)	*Zea mays*	−	+	23
M-Maiz2 (CFBP 5904[d])	France, Maine valley (1992)	*Zea mays*	−	+	23
M-Maiz3 (CFBP 5911[e])	France, Maine valley (1992)	*Zea mays*	−	+	23
M-Maiz4 (CFBP 5914[f])	France, Maine valley (1992)	*Zea mays*	−	+	23
A-Tom1 (CFBP 5926[g])	France, Angers (1992)	*Lycopersicon esculentum*	−	+	23
A-Tom2 (CFBP 5932[h])	France, Angers (1992)	*Lycopersicon esculentum*	−	+	23
C-Barr1 (MTR-48-522)	France, Châteaurenard (2009)	*Medicago trunculata*	−	+	49
C-Barr2 (MTR-J5-721)	France, Châteaurenard (2009)	*Medicago trunculata*	−	+	49
C-Barr3 (MTE-48-204)	France, Châteaurenard (2009)	*Medicago trunculata*	−	+	49
Plant pathogen reference strain *P. syringae* DC3000	Canada (1985)	*Lycopersicon esculentum*	−	+	95

[a] Detection of T3SS genes is highlighted in gray to facilitate reading.
[b] CFBP, French Collection of Plant-associated Bacteria.
[c] Previous name in the collection, CFBP11348.
[d] Previous name in the collection, CFBP11355.
[e] Previous name in the collection, CFBP11364.
[f] Previous name in the collection, CFBP11367.
[g] Previous name in the collection, CFBP11386.
[h] Previous name in the collection, CFBP11393.

the total number of RBCs lysed, obtained by incubating cells with 0.1% (wt/vol) SDS. *X* is the OD value of the analyzed sample.

***Dictyostelium discoideum* growth inhibition assay.** The amoeba *D. discoideum* can be used in a model pathosystem (59). This eukaryotic cell is a soil amoeba that feeds on bacteria by phagocytosis. *D. discoideum* colonizes the same niches as numerous bacteria, including *Pseudomonas*. This amoeba shares many specific functions with mammalian phagocytic cells, like macrophages. A large number of bacterial virulence traits, including the capacity to counteract the immune response, can be studied using *D. discoideum* (60). *D. discoideum* forms visible lysis plaques when grown on a bacterial layer as the feeding source in petri dishes. If the bacteria are virulent to *D. discoideum*, they cause amoeba growth inhibition and/or death, which can be observed by a decrease in the number of lysis plaques. In pathogenic *P. aeruginosa* and *P. fluorescens* isolates, *D. discoideum* growth inhibition is the result of a T3SS and/or biosurfactant contact (60, 61). This test was performed as described by Carilla-Latorre et al. (96) (62). Briefly, *D. discoideum* AX3 cells were grown axenically in HL5 medium at pH 6.5 (Formadium) or in association with *K. aerogenes* on skim milk (SM) agar (63) plates at pH 6.5 (Formadium). For the nutrient SM-plating assay, bacterial strains were grown overnight in KB medium at 25 or 37°C. *P. aeruginosa* PA14 was used as a positive control for *D. discoideum* growth inhibition and *K. aerogenes* as a negative control. *K. aerogenes* was used as a nutritive strain and was grown overnight in LB medium at 37°C. After washing in HL5, *Pseudomonas* strains were resuspended in HL5 to obtain an OD_{580} of 1. The *K. aerogenes* density was adjusted to an OD_{580} of 0.5. Three hundred microliters of *K. aerogenes* cell

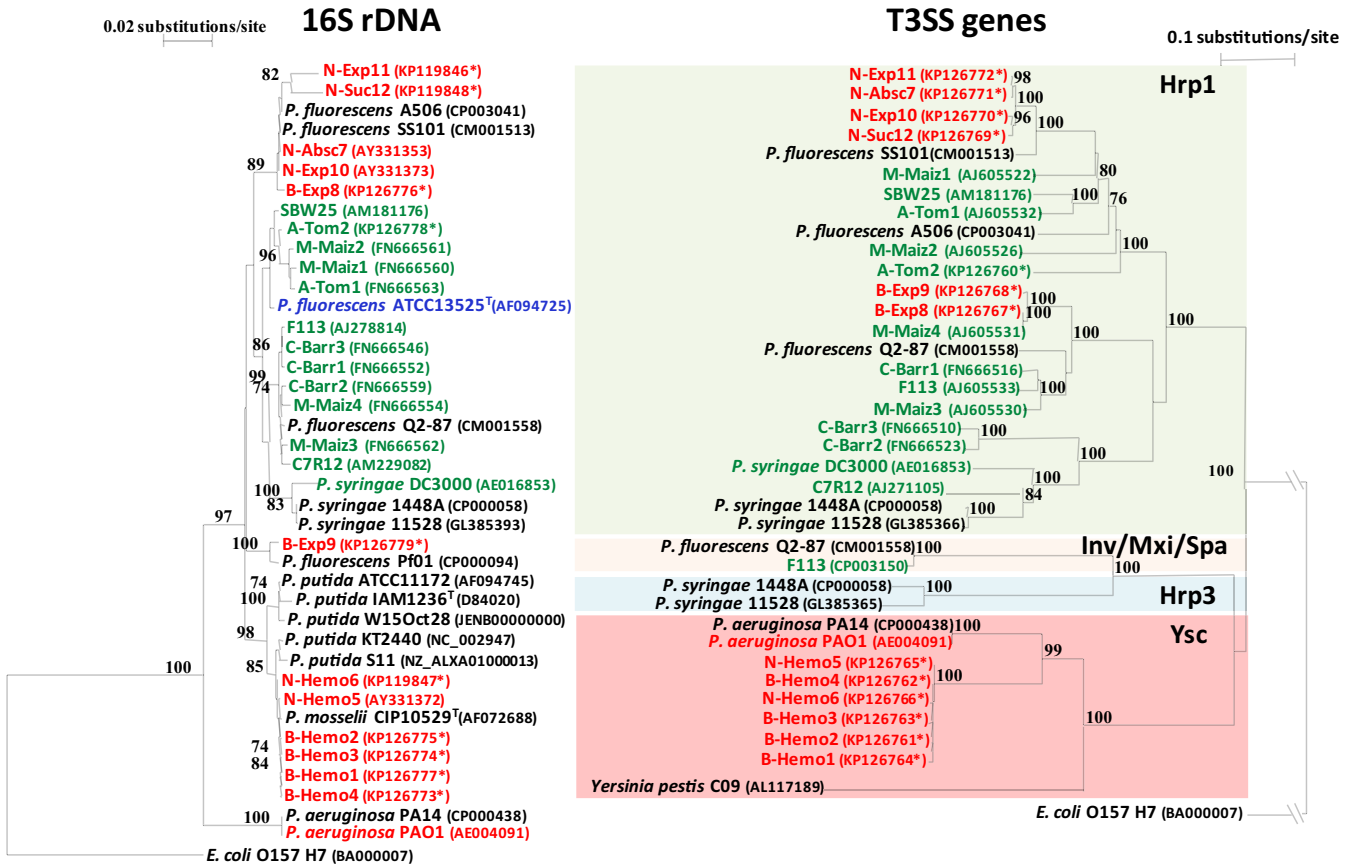

FIG 1 Phylogenetic relationships between clinical and plant-associated pseudomonads harboring T3SS genes based on DNA sequences of 16S rRNA gene and of T3SS genes closely related to *hrc* (or *rsc*) and *pscRST*. Names of clinical isolates, plant-associated isolates, *P. fluorescens* type strain, and reference sequences found in GenBank are written in red, green, blue, and black font, respectively. The number shown next to each node indicates the percentage bootstrap values of 1,000 replicates that exceeded 70%. The database accession numbers are given in parentheses. *, sequences obtained in the present study. A superscript T indicates the type strain. Families of T3SS named as defined by Loper et al. (16) are indicated in bold in the top right corners of the colored boxes. The 16S rRNA gene and *escRST* sequences of *Escherichia coli* O157H7 were used to root both phylogenetic trees.

suspension and 15 μl of *Pseudomonas* cell suspension (ratio, 10%) were plated on SM agar with approximately 100 *D. discoideum* cells. The plates were kept at 22°C for 5 days, after which the lysis plaques were counted. The results were expressed as percentages of *D. discoideum* growth inhibition. These values were calculated as follows: $\% = (K - X - P)/X$, where K is the number of lysis plaques observed on plates containing the positive-control strain *K. aerogenes* (approximately 100), X is the number of lysis plaques on plates containing the tested strain, and P is the number of lysis plaques obtained for the negative-control strain *P. aeruginosa* PA14 (which was 0 in all our experiments).

Biosurfactant production assay. Biosurfactants are small molecules secreted by numerous bacteria and able to induce the rapid lysis of eukaryotic cells. Among these, the rhamnolipids secreted by *P. aeruginosa* strain PAO1 could induce rapid lysis of *D. discoideum* cells (59). Biosurfactant production was assessed by the drop-collapse test as described previously (64). Briefly, drops of mineral water (Volvic, France) were dispensed into a polystyrene petri dish. One drop of supernatant obtained by centrifugation (5 min at 6,000 × *g*) of bacterial cells in stationary growth phase in KB broth was added to 1 drop of water. The result of the test was considered positive when the water drop collapsed.

Chicory leaf model of infection. The chicory leaf test was performed as described by Fito-Boncompte et al. (65). Briefly, NaCl-washed cell suspensions (cell density, ca. 10^8 CFU/ml in 0.9% [wt/vol] NaCl) were prepared from stationary-phase cultures grown in KB medium. Chicory (*Cichorium intybus*) leaves were surface sterilized with 0.1% bleach and then infected by injecting 10 μl of the above-described bacterial suspension into the midrib. The leaves were placed on petri dishes and incubated in a Minitron incubator (Infors, Massy, France) at 25 or 37°C under a relative humidity of 65% ± 2%. For each strain, three leaves were analyzed and the development of the symptoms was evaluated after 7 days.

HR in tobacco plants. Induction of the hypersensitive response (HR), a local programmed death of plant cells at the infection site, suggests that effector proteins are translocated into the plant cell via T3SSs (66–68). Thus, an HR can be generated only if the bacterium harbors an operational T3SS and injects effector proteins into the plant cell. This is the action mode of the hemibiotroph *P. syringae* DC3000, which requires living host tissue as part of the infection process, during which it produces at least 38 putative effector proteins (51, 69, 70). This test was performed as adapted by Smadja et al. (96). Briefly, tobacco (*Nicotiana tabacum* cv. Xanthi) XHFD8 was grown for 8 weeks under greenhouse conditions (temperature, 25°C; photoperiod, 16 h). Bacteria were grown in KB liquid medium at 25°C, washed twice with 0.9% (wt/vol) NaCl, and resuspended at 2×10^9 CFU/ml. Tobacco leaves were infiltrated with ca. 100 μl of cell suspensions. The margins of the water-soaked infiltrated areas were marked, and the plants were inspected for HR development after 24 and 48 h.

Nucleotide sequence accession numbers. The sequences newly determined in this study were submitted to GenBank under the accession numbers KP119846 to KP119848 and KP126760 to KP126779 (shown in Fig. 1 with asterisks).

RESULTS

Detection and analysis of the T3SS *hrp* (*rsc*) and *psc* gene homologues. The 12 hospital isolates were screened for the presence of three T3SS marker genes designated *hrcR*, *hrcS*, and *hrcT* or *rscR*, *rscS*, and *rscT* in strains carrying Hrp1-T3SS clusters. New primers were designed, and the PCR method was then adapted to amplify the corresponding sequences of *pscR*, *pscS*, and *pscT* of *P. aeruginosa*. The latter method was then applied to screen all strains for the presence of sequences homologous to *psc* genes. As expected, the *P. fluorescens* ATCC 13525^T type strain was negative for both PCRs, the reference strain *P. aeruginosa* PAO1 was PCR positive for *pscRST* and negative for *hrcRST* (*rscRST*), and the plant reference strains *P. syringae* DC3000, *P. fluorescens* C7R12, and *P. fluorescens* SBW25 were PCR negative for *pscRST* and positive for *hrcRST* (*rscRST*). The plant reference strain *P. fluorescens* F113 was the only strain positive for both PCRs.

Two groups were distinguished within the clinical isolates. The first group consisted of all isolates originating from patients with respiratory tract infections and harbored sequences homologous to those of *hrcRST* (*rscRST*). The second group consisted of all the isolates obtained from patients with blood infections and harbored sequences homologous to those of *pscRST* (Table 1).

Sequence analysis of the amplified DNA fragments in the first group confirmed the presence of Hrp1-T3SS genes in the respiratory tract isolates. Phylogenetic analysis showed that the *hrcRST* (*rscRST*) sequences amplified from these isolates fell into a clade that encompassed all the environmental and reference strains harboring Hrp1-T3SS (Fig. 1). Four of these six isolates (N-Exp10, N-Exp11, N-Suc12, and N-Absc7) displayed the highest identity (94%) with *rscRST* genes from the biocontrol agent *P. fluorescens* SS101 (16) and were grouped together in a subclade that also included five of the plant strains (M-Maiz1, M-Maiz2, A-Tom1, A-Tom2, and SBW25) and *P. fluorescens* A506 (16). The last two isolates (B-Exp8 and B-Exp9) were grouped in a second subclade that included the biocontrol strain *P. fluorescens* Q2-87 (16) and four of the plant-associated strains studied, *P. fluorescens* F113, C-Barr1, M-Maiz3, and M-Maiz4. Both hospital isolates shared the highest sequence identity (99%) with the last strain.

All the T3SS sequences amplified from blood isolates in the second group were very closely related (identity, 99 to 100%). Although identities shared with *P. aeruginosa* strains remained quite moderate (78 to 79%), they represented the second-highest percentages observed. The phylogenetic representation showed that, like the *P. aeruginosa* strains, isolates from this second group belonged to the Ysc-T3SS family, as indicated by the high bootstrap values (Fig. 1). The highest identity (86%) was observed between T3SS sequences amplified from blood isolates and sequences belonging to the newly released genome of *Pseudomonas mosselii* SJ10 (CP009365) isolated from industrial wastewater.

Genetic relationships of fluorescent pseudomonads harboring T3SS genes. Analysis of the 16S rRNA sequences confirmed the close relationship between strains belonging to *P. fluorescens* and the hospital isolates originating from respiratory tract infections. For these strains, the closest relatives found in databases were strains of *P. fluorescens* belonging to the *P. fluorescens* complex as described by Yamamoto et al. (71) and to the *P. fluorescens* group as described by Anzai et al. (72). A phylogenetic tree, which included representatives of the closest relatives to the clinical isolates studied found in databases (Fig. 1), showed that hospital isolate B-Exp9 was closely related to *P. fluorescens* Pf01, whereas the five other isolates belonged to a different group, together with the biocontrol agents *P. fluorescens* SS101 and A506. Nevertheless, T3SS genes and 16S rRNA gene phylogenies stressed distinct relationships among isolates from respiratory tract infections with sequences of T3SS genes in B-Exp9 and B-Exp8 being closely related and those of 16S rRNA gene being distant. In contrast, 16S rRNA sequences of all hospital isolates originating from blood infections were very closely related (identity, 99 to 100%), as were T3SS gene sequences; 16S rRNA phylogeny also revealed a close relationship (identities, ≥99%) of these blood isolates with *Pseudomonas putida* and *Pseudomonas mosselii* strains within a clade (Fig. 1) previously designated "*P. putida* complex" (71) or "*P. putida* group" (72).

Phenotypic traits. (i) Bacterial growth temperature range. All collected *Pseudomonas* strains were grown in liquid and on solid surfaces to check their ability to live under environmental conditions (between 4 and 25°C) and to highlight their ability to multiply at human physiological temperature (37°C) and resist febrile episodes (slight or strong transition at 41°C). All the clinical isolates were able to grow at 37°C, and this ability was also observed for some of the environmental strains, i.e., 10 of 12 strains on agar and 4 of 12 in liquid medium (Table 2). These strains kept their psychrotrophic characters (growth at 4°C, optimal growth temperature around 25°C). All isolates originating from blood infections were resistant to strong transition up to 41°C, an observation concomitant with their lack of multiplication at 4°C (Table 2).

(ii) Cell-associated hemolytic activity. The cHA assay evaluates the ability of a bacterial cell to perforate or hydrolyze an animal cell membrane after moderate contact enhanced by gentle centrifugation, which avoids disruption of the long and thin structures on the bacterial cell surface. All clinical strains displayed cell-associated hemolytic activity (Fig. 2). The blood isolates, i.e., strains B-Hemo 1 to N-Hemo 6, showed weak cHA (less than or equal to 20% after growth at 25°C or 37°C). Most of the respiratory tract isolates, except B-Exp8, exhibited cHA of >40% for at least one growth temperature, i.e., strains B-Exp 9, N-Exp 10, N-Exp 11, and N-Suc12. The N-Absc7 strain, isolated from an abscess, showed high cHA, above 60%, at both growth temperatures. As expected, none of the strains isolated from plants displayed cHA significantly different from that of the negative control under the conditions used for the bioassay.

(iii) Amoebic growth inhibition. All clinical isolates induced *D. discoideum* growth inhibition for at least one growth temperature (Fig. 3), most of them (9 of 12) being virulent against *D. discoideum* at both temperatures tested. Except for N-Exp10, which was able to reduce *D. discoideum* growth only at 25°C, the inhibition induced by all clinical isolates at 37°C was greater than or equal to that recorded for PAO1 (Fig. 3).

Among the plant isolates, only M-Maiz4 was able to inhibit *D. discoideum* lytic activity at both growth temperatures. Two strains, C7R12 and C-Barr2, inhibited lysis plaque production only at 37°C and four (F113, SBW25, ATCC 13525^T, and C-Barr3) did so only at 25°C.

(iv) Biosurfactant production. PAO1 did not produce any measurable biosurfactant under our conditions. In five cases, biosurfactant activity was detected without *D. discoideum* growth in-

TABLE 2 Growth temperature range of fluorescent pseudomonads[a]

Strain	Growth temp and culture medium									
	4°C		25°C		37°C		41°C AGC[b]		41°C	
	Agar	Liquid	Agar	Liquid	Agar	Liquid	Agar	Liquid	Agar	Liquid
Human pathogen reference strain *P. aeruginosa* PAO1	−	−	+	+	+	+	+	+	+	+
Pseudomonas fluorescens clinical isolates										
B-Hemo1	−	−	+	+	+	+	+	+	+	+
B-Hemo2	−	−	+	+	+	+	+	−	−	−
B-Hemo3	−	−	+	+	+	+	+	−	−	−
B-Hemo4	−	−	+	+	+	+	+	−	−	−
N-Hemo5	−	−	+	+	+	+	+	+	−	−
N-Hemo6	−	−	+	+	+	+	+	+	−	−
N-Absc7	+	+	+	+	+	+	−	−	−	−
B-Exp8	−	−	+	+	+	+	+	−	−	−
B-Exp9	+	+	+	+	+	+	−	−	−	−
N-Exp10	+	+	+	+	+	+	−	−	−	−
N-Exp11	+	+	+	+	+	+	−	−	−	−
N-Suc12	+	+	+	+	+	+	−	−	−	−
Pseudomonas fluorescens type strain ATCC 13525[T]	+	+	+	+	−	+/−	−	−	−	−
Pseudomonas fluorescens plant-associated isolates										
C7R12	+	+	+	+	+	+	−	−	−	−
F113	+/−	+	+	+	−	−	−	−	−	−
SBW25	+	+	+	+	+	−	−	−	−	−
M-Maiz1	+	+	+	+	+	−	−	−	−	−
M-Maiz2	+	+	+	+	−	−	−	−	−	−
M-Maiz3	+/−	+	+	+	+	−	−	−	−	−
M-Maiz4	+	+	+	+	+	+	−	−	−	−
A-Tom1	+	+	+	+	+	−	−	−	−	−
A-Tom2	+	+	+	+	+	−	−	−	−	−
C-Barr1	+	+	+	+	+	−	−	−	−	−
C-Barr2	+	+	+	+	+	+	+	−	−	−
C-Barr3	+	+	+	+	+	+	−	−	−	−
Plant pathogen reference strain *P. syringae* DC3000	+	+	+	+	−	−	−	−	−	−

[a] Symbols: +, growth; +/−, impaired growth; −, no growth on King B medium. Positive growth test results are highlighted in gray in order to facilitate reading.
[b] AGC, adaptive growth culture conditions based on an initial *Pseudomonas* preculture at 25°C prior to the test culture at 41°C.

hibition, and most of the isolates virulent against *D. discoideum* did not produce biosurfactant (Fig. 3).

(v) Plant virulence on chicory leaves. *P. syringae* DC3000 induced a slight maceration at the point of infiltration after 3 days at 25°C (Fig. 4A). *P. aeruginos*a induced black necrosis at the site of inoculation in the middle vein of each leaf after 3 days, but only at 37°C (Fig. 4B). None of the other *Pseudomonas* isolates tested (harboring or not T3SS genes) induced symptoms on chicory leaves at 25 or 37°C.

(vi) Induction of plant defense mechanisms. Only three plant-associated *P. fluorescens* strains (C7R12, F113, and M-Maiz4) induced an HR on tobacco leaves comparable to that recorded for phytopathogenic *P. syringae* DC3000 (Fig. 4C and D).

DISCUSSION

Bacteria identified as *P. fluorescens* are generally considered safe and devoid of any pathogenic potential (2). Nevertheless, the recurrent isolation of *P. fluorescens* from clinical cases in hospitals is raising concerns about the possible emergence of pathogenic strains that could be particularly resistant to the immune system and clinical treatments (38, 39, 73). This concern has been further augmented by the observed large genome size and plasticity of these bacteria, which allows a high potential adaptation to changing environments and could facilitate opportunistic pathogenic behavior. In this context, it is interesting that T3SSs, known to be involved in the virulence of *P. aeruginosa* and *P. syringae* via the injection of effector proteins into the host cells, have also been found in plant-associated *P. fluorescens*. Taken together, these observations raise questions about the presence of T3SSs in clinical *P. fluorescens* and the possible contribution of these secretion systems in potential virulence of the corresponding isolates.

The aim of the present study was therefore to assess (i) the possible presence of T3SS in clinical *P. fluorescens*, (ii) the similar-

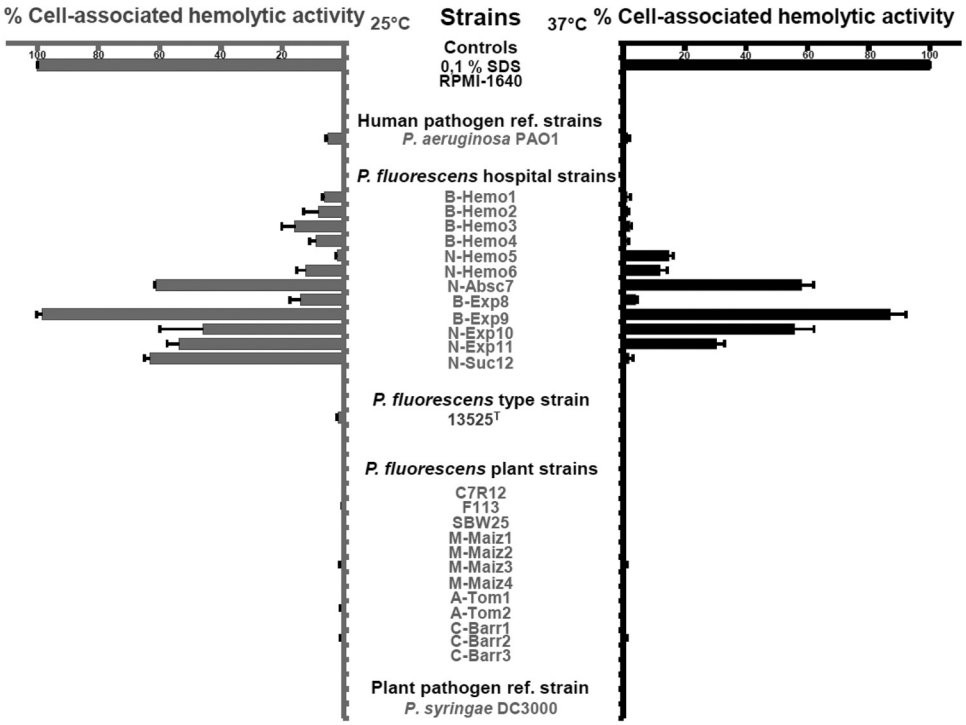

FIG 2 Cell-associated hemolytic activity of fluorescent pseudomonads strains harboring T3SS genes. Cell-associated hemolytic activity was measured as described in Materials and Methods. Results are mean values from at least three independent experiments. Standard deviations are shown. Red blood cells were incubated 1 h at 37°C with bacteria grown at 25 or 37°C (multiplicity of infection, 1). nd, not determined (strains did not grow at 37°C).

ities of these T3SSs with those in plant-associated *P. fluorescens*, and (iii) the relationship between the presence of T3SSs in human- and plant-associated *P. fluorescens* strains and their potential virulence.

Data from the present study revealed strong similarities between the human isolates originating from respiratory tract infections and plant-associated isolates. They all harbored T3SS genes belonging to the Hrp1-T3SS family, thereby confirming the widespread occurrence of these T3SSs in *P. fluorescens* strains (16, 23, 49). Furthermore, they could not be differentiated on the basis of the phylogeny of their 16S rRNA or T3SS genes sequences. They fell into groups belonging to the *P. fluorescens* complex, as described by Yamamoto et al. (71) (Fig. 1). Similarly, the two groups of T3SS sequences identified in the respiratory tract isolates (Fig. 1) were closely related to groups previously described for plant-associated isolates (16, 49). As previously reported for plant-associated isolates (23, 49), the phylogeny of the T3SS genes in clinical isolates from the respiratory tract was not congruent with that of the 16S rRNA gene. In contrast, a clear congruence was found between the geographic origin of these clinical isolates (Franche-Comté or Normandy) and the group of T3SS sequences, whereas no relationship was apparent between 16S rRNA phylogeny and geographical origin. These observations could suggest possible gene acquisition by horizontal transfer, as previously reported in pseudomonads (74). The respiratory tract and plant-associated isolates also showed similarities in their phenotypic traits and virulence potential as assessed by the *D. discoideum* assay. All isolates, except one respiratory tract isolate, were able to grow at low temperature. No bacterial virulence factor able to modify plant cells was detected in respiratory tract and plant-associated isolates. This could be associated with the fact that all the respiratory tract isolates and most of the plant-associated isolates were unable to induce any HR despite the presence of Hrp1-T3SS genes. Nor could the respiratory tract and plant-associated isolates be differentiated on the basis of their virulence against *D. discoideum* or their ability to grow at high temperatures, since these traits were shared by all human isolates and were also found in some of the plant-associated isolates. Respiratory tract and plant isolates could be differentiated only on the basis of cell-associated hemolytic activity, which was expressed by human isolates but not by plant isolates. Since all clinical isolates harbored T3SS and since human- and plant-associated isolates could be differentiated only for hemolysis and not for the phylogeny of the T3SS genes, the potential virulence of *P. fluorescens* to animal cells would not be related to their origin (plant or human) but rather to the presence of T3SSs and would therefore support the hypothesis of an opportunistic behavior of *P. fluorescens*. Thus, the plant rhizosphere, already known for its ability to host populations known to be human pathogenic and belonging to *P. aeruginosa* (75, 76), *Burkholderia* spp. (77, 78), or *Escherichia coli* (79), could also be a potential source of *P. fluorescens* opportunistic pathogens. The possible involvement of Hrp1 family secretion systems in the virulence of respiratory tract isolates toward animal cells is supported by the altered virulence of a mutant of the respiratory tract isolate *P. fluorescens* MFN1032 (syn. N-Exp11 in our study), which had an impaired T3SS (58, 61). However, this virulence cannot solely be ascribed to T3SS since no virulence potential against animal cells was expressed by 6 of the 12 plant-associated isolates, despite the presence of Hrp1-T3SS. Identification of the virulence determinants of clinical *P. fluorescens* isolates could be

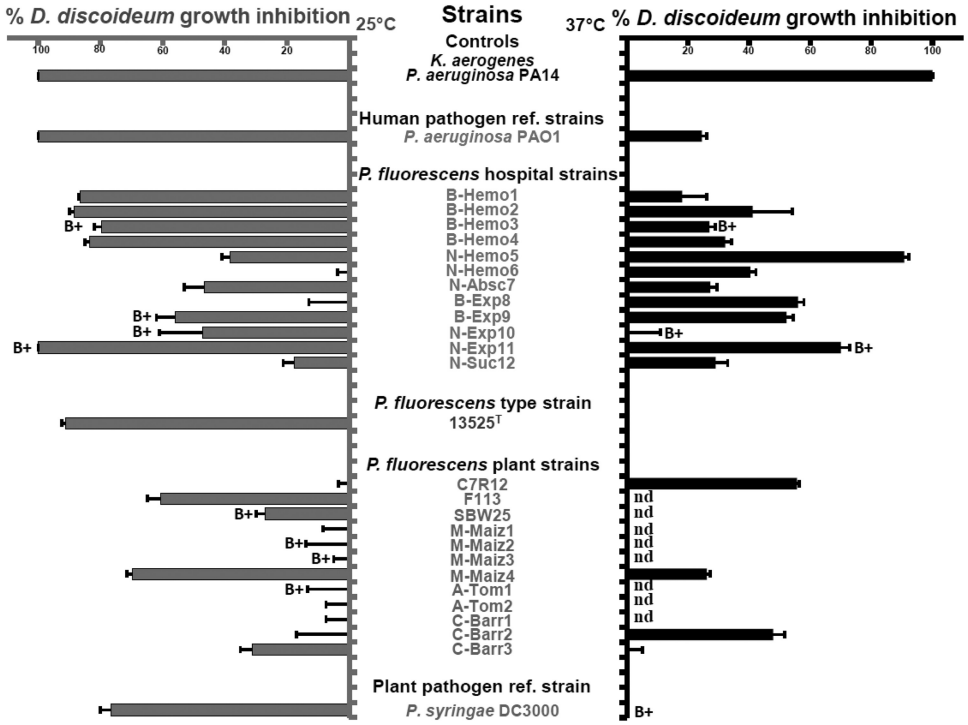

FIG 3 *Dictyostelium discoideum* lysis induced by fluorescent pseudomonads strains harboring T3SS genes and biosurfactant production. *Dictyostelium discoideum* growth inhibition was calculated as described in Materials and Methods with bacteria grown at 25 or 37°C. Data are mean values from three independent experiments, and standard deviations are shown. Bacterial biosurfactant production was estimated by the drop collapse method as described in Materials and Methods. B+ indicates drop collapse and therefore biosurfactant production. nd, not determined (strains did not grow at 37°C).

aided by comparing the putative T3SS effector proteins secreted by these 6 plant isolates with those detected in plant and human isolates capable of virulence against animal cells. In this context, an additional T3SS belonging to the Inv/Mxi/Spa family (also known as SPI-1), so far described only in human- and animal-associated bacteria, has recently been reported in two plant-associated *P. fluorescens* strains (16, 80, 81). In the present study, one of these strains (F113) gave a weak positive signal in the *pscRST* PCR, which could possibly be related to the presence of Inv/Mxi/Spa-T3SS genes (82, 83) that appeared to show high sequence identity with the *psc* sequences targeted by our primers (data not shown). Interestingly, the plant-associated strain F113, harboring Inv/Mxi/Spa-T3SS genes, was shown here to express virulence against animal cells.

In contrast to what was seen with the respiratory tract isolates, the clinical isolates originating from blood infections could clearly be differentiated from plant-associated *P. fluorescens*. This group of clinical isolates was highly homogeneous. Except for surfactant production, all representatives displayed the same characteristics irrespective of their geographical origin. The phylogeny of their 16S rRNA sequences indicated that they shared very high levels of identity and belonged to the *P. putida* complex as defined by Yamamoto et al. (71). The original assignation of these isolates to *P. fluorescens* can be explained by the fact that the standard phenotypic traits habitually used for species identification in hospitals do not allow a clear differentiation between the species *P. fluorescens* and *P. putida* (4). These findings are in agreement with previous reports on clinical strains isolated from three distinct regions (Normandy, Paris-Ile de France, and North of France) that were first identified, according to phenotypic traits, as belonging to *P. fluorescens* but were then shown to include a homogeneous group taxonomically distinct from *P. fluorescens* (84, 85). A new species, *P. mosselii*, was proposed for isolates belonging to this group (84). Bodilis et al. (85) confirmed the presence of this homogeneous group and showed that the corresponding isolates, which included MFY161 (syn. N-Hemo5 in our study), belonged to the *P. putida* complex (71) together with the *P. mosselii* type strain. The very high similarity between N-Hemo5 and other clinical isolates from blood infections observed in the present study shows that they all belong to the same above-mentioned bacterial group. Four of our isolates originated from a hospital in Franche-Comté, thus extending the detection of this specific group of pseudomonads to an additional region. This observation suggests a more widespread occurrence of bacteria belonging to this group in clinical isolates and regularly misidentified in hospitals as *P. fluorescens* (84, 85). Isolates from the *P. putida* complex were shown here to express virulence against animal cells and to grow at high temperatures (37 and 41°C), further supporting the hypothesis proposed by Dabboussi et al. (84), McLellan and Partridge (86), and Leneveu-Jenvrin et al. (87) concerning the human pathogenicity of this group of bacteria, whereas they would have been classified as nonpathogenic or at most opportunistic bacteria on the basis of the standard taxonomic identification applied in hospitals. Furthermore, isolates from this group were also characterized by the presence of T3SS genes belonging to the Ysc-T3SS family, which have so far been reported only in human and animal pathogens and, within *Pseudomonas*, only in *P. aeruginosa*. Although they appear to be close relatives, the *pscRST* genes of *P.*

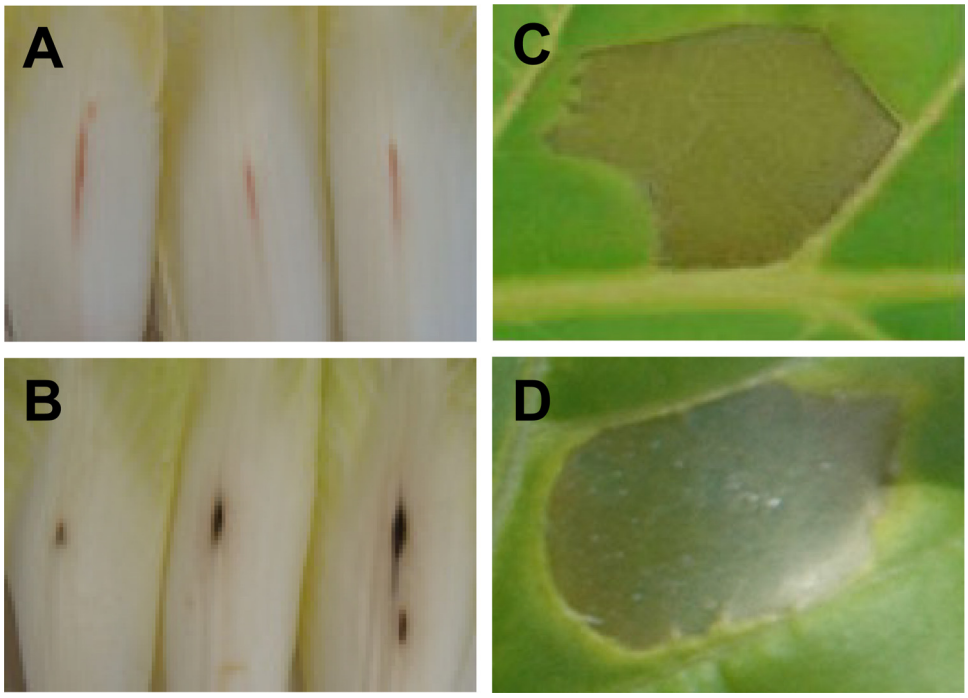

FIG 4 Symptoms in plant induced by fluorescent pseudomonad strains harboring T3SS genes. Induction of soft rot by *P. syringae* DC3000 at 25°C (A) and necrosis at 37°C by *P. aeruginosa* PAO1 (B) in chicory leaves (*Cichorium intybus*). Leaf panels were infiltrated in the middle vein with cells of each *Pseudomonas* strain at a concentration of 10^8 CFU/ml in 0.9% (wt/vol) NaCl. Pictures were taken 72 h after infiltration and are representative of at least three independent experiments. Elicitation of a hypersensitive response in tobacco leaves (*Nicotiana tabacum* cv. Xanthi) at 25°C by *P. syringae* DC3000 (C) and *P. fluorescens* C7R12 (D). Leaf panels were infiltrated with cells of each *Pseudomonas* strain at a concentration of 2×10^9 CFU/ml in 0.9% (wt/vol) NaCl. Pictures were taken 24 h after infiltration and are representative of at least three independent experiments.

aeruginosa could be clearly distinguished from the sequences described here. Therefore, we report here for the first time the presence of a T3SS related to the well-studied T3SS of *P. aeruginosa* (17–19) in isolates belonging to the *P. putida* complex. This finding is strengthened by results of homology searches in data banks. Indeed, they stressed the presence of sequences closely related to T3SS genes of the clinical isolates from blood infections in another strain of *Pseudomonas* belonging to the *P. putida* complex, SJ10 (CP009365), isolated from a different environment (industrial wastewater). The potential virulence of the clinical isolates from the *P. putida* complex, as indicated by hemolytic activity and *D. discoideum* inhibition, would imply the presence of a functional T3SS. Moreover, newly described T3SS genes have been found to be highly conserved irrespective of their geographic origin, which is a common feature of host-adapted pathogens (88, 89). Therefore, the T3SS genes newly described here could be good candidates for the development of a simple and rapid PCR detection assay for identifying bacteria belonging to the *P. putida* complex. Determining the occurrence of the corresponding putative pathogens in hospitals, so far possibly underestimated due to lack of an appropriate test (86), could facilitate diagnosis.

In conclusion, at least one secretion system was detected in isolates showing virulence against animal cells. T3SS genes were detected in all clinical isolates belonging to *P. fluorescens* (from respiratory tract infections) and to the *P. putida* complex (from blood infections). Hrp1-T3SSs genes from human and plant *P. fluorescens* could not be discriminated. The presence of T3SS genes of the Ysc family in *P. putida* complex bacteria is reported here for the first time. Taken together, these findings suggest that the relationship between T3SSs and virulence, well described in *P. aeruginosa* and *P. syringae*, could be extended to *P. fluorescens* and *P. putida* bacteria. Finally, the presence of T3SS genes related to those of *P. aeruginosa* in clinical isolates belonging to the *P. putida* complex, together with their association with bacteremia and with phenotypic traits indicating their possible virulence in humans, urges further study of this bacterial group, which is commonly misidentified in hospitals as *P. fluorescens*. This could be achieved by the development of a rapid identification test based on the presence of characteristic T3SS genes as identified in the present study. In contrast, clinical *P. fluorescens* could not be differentiated from environmental strains belonging to this species, and no genomic trait could therefore be identified to discriminate clinical and environmental isolates. Altogether, comparative genomic studies on environmental and clinical strains focusing on T3SSs and mediated effector molecules would help for further elucidation of pathogenicity determinants among *P. fluorescens* and *P. putida*.

ACKNOWLEDGMENTS

This research was funded by grants from the Conseil Régional de Haute-Normandie & Ministère délégué à l'Enseignement Supérieur et à la Recherche, Réseau VASI (GRR TERA) & FEDER. The study was supported by the Groupement de Recherche CNRS *Pseudomonas* and by IOBC-WPRS. Work was related to COST631 action "Understanding and Modeling Plant-Soil Interactions in the Rhizosphere Environment" and to the Association of Applied Biology.

We are grateful to Thérèse Corberand for technical assistance and to Diana Warwick for correcting the English text.

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